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Prevalence and age-related variation of *Cryptosporidium* species and genotypes in dairy calves

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Abstract

Fifteen dairy farms in seven states on the east coast of the US were each visited on two consecutive years to determinate the prevalence of *Cryptosporidium* species in pre-weaned (5 days to 2 months) and post-weaned calves (3–11 months), respectively. After each of 971 fecal specimens collected directly from each calf was sieved and subjected to density gradient centrifugation to remove debris and concentrate oocysts, specimens were examined by immunofluorescence microscopy, and polymerase chain reaction (PCR). For all PCR-positive specimens the 18S rRNA gene of *Cryptosporidium* was sequenced. *Cryptosporidium* was identified from all farms. Types of housing appeared to have no influence with regard to prevalence of infection. Of 971 calves, 345 were infected with *Cryptosporidium* (35.5%), but more pre-weaned calves (253 of 503; 50.3%) than post-weaned calves (92 of 468; 19.7%) were found to be infected. A total of 278 PCR-positive specimens characterized by gene sequencing revealed *Cryptosporidium parvum*, *Cryptosporidium andersoni*, and two unnamed *Cryptosporidium* genotypes Bovine B (AY120911) and deer-like genotype (AY120910). The prevalence of these *Cryptosporidium* species and genotypes appeared to be age related between pre- and post-weaned calves. *C. parvum*, the only zoonotic species/genotype, constituted 85% of the *Cryptosporidium* infections in pre-weaned calves but only 1% of the *Cryptosporidium* infections in post-weaned calves. These findings clearly demonstrate that earlier reports on the presence and prevalence of *C. parvum* in post-weaned cattle that were based solely on oocyst morphology must

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be reassessed using molecular methods to validate species and genotype. This finding also indicates that persons handling or otherwise exposed to calves under 2 months of age are at greater risk of zoonotic infection from *Cryptosporidium* than the risk of infection from exposure to older calves.

Keywords: *Cryptosporidium*; Prevalence; Calves; Genotyping; Zoonoses

1. Introduction

Cryptosporidial infection is one of the most common causes of diarrhea in humans and livestock worldwide (Webster, 1993; Casemore et al., 1997). Using morphological criteria, host specificity, and DNA-based studies, 13 species have been recognized in the genus *Cryptosporidium* (Monis and Thompson, 2003; Xiao et al., 2004). Molecular studies have provided considerable evidence of genetic heterogeneity among isolates of *Cryptosporidium* from different species of vertebrates, and there is now evidence suggesting that a series of host-adapted genotypes/species of the parasite exist (Morgan et al., 1999; Fayer et al., 2000a; Xiao et al., 2002, 2004). Genotypes of *Cryptosporidium*, each associated with specific animal hosts include goose genotypes, duck genotype, pig genotype, skunk genotype, ferret genotype, muskrat genotypes I and II, *C. canis* fox genotype (Xiao et al., 2002; Enemark et al., 2003; Ryan et al., 2003; Jellison et al., 2004).

Two species of *Cryptosporidium* with significant biological differences infect cattle: *C. parvum* and *C. andersoni* (formerly known as *C. muris*; Lindsay et al., 2000). *Cryptosporidium parvum*, a pathogen that infects the intestine of young calves, humans, and other animals, often results in acute enteritis and diarrheal disease (Casemore et al., 1997; Fayer et al., 1997, 2000a; Morgan et al., 1999) whereas *C. andersoni*, possibly associated with reduced milk production, infects the abomasum of juvenile and mature cattle, and is neither associated with overt clinical signs nor is known to infect animals other than cattle (Olson et al., 1997; Anderson, 1998; Lindsay et al., 2000). Recently, a new genotype of *Cryptosporidium* was found in cattle and was identified as *Cryptosporidium* Bovine genotype B (Xiao et al., 2002).

Many studies of *Cryptosporidium* in cattle have been based either on morphological identification of oocysts in feces (Quílez et al., 1996; Uga et al., 2000; Wade et al., 2000; Zorana et al., 2001; Castro-Hermida et al., 2002a,b; Kvac and Vitovec, 2003) or immunofluorescence microscopy using non-species-specific antibody (Xiao et al., 1993; Xiao and Herd, 1994; Olson et al., 1997; Atwill et al., 1999; O'Handley et al., 1999; Fayer et al., 2000b; Sischo et al., 2000; Ralston et al., 2003; Sturdee et al., 2003). Although both methods can provide generalized prevalence data for the presence of *Cryptosporidium*, neither method is sufficient to identify species or genotypes of *Cryptosporidium* (Fayer et al., 2000a; Egyed et al., 2003; Monis and Thompson, 2003). The present study was undertaken: (a) to expand the geographic range of calves examined from single farms or multiple farms within a state or region to a multi-state area encompassing much of the east coast of the US, (b) to increase the number of animals examined from a herd or specific age group to nearly 1000 pre-weaned and post-weaned calves, and (c) to precisely identify the species and genotypes of *Cryptosporidium* oocysts found by utilizing molecular techniques.

2. Material and methods

2.1. Sources and collection of specimens

Feces were collected from 971 calves, located on three dairy farms in Pennsylvania and two dairy farms each in Vermont, New York, Maryland, Virginia, North Carolina, and Florida (Table 1). All farms were visited twice, once in 2002 and again in 2003, except the Pennsylvania farms PA-2 and PA-3 that were visited just once (PA-2 in 2002; PA-3 in 2003). The number of calves per farm that provided fecal specimens is shown in Table 1. The only selection criterion for farms was the ability to provide a minimum of 15 calves of the appropriate age for sampling during each visit.

In 2002 fecal specimens were obtained from calves from 5 days to 2 months of age (pre-weaned calves). In 2003 fecal specimens were obtained from calves between 3 and 11 months old of age (post-weaned calves), none of which were sampled in 2002. From here on we will referred to these two age groups as pre- and post-weaned calves. Age-related data were examined on the basis of pre-weaned versus post-weaned status (Tables 1–3) and were examined as 14 age groups, weekly during the first month of age and monthly thereafter (Fig. 1C). Age-related data were not available for VT-2, in 2002 and 2003, and for VT-1, PA-3, and VA-1 in 2003.

Table 1
Specimens examined for *Cryptosporidium* by IFA from each farm examined

State	Farm	Pre-weaned calves		Post-weaned calves	
		No. of specimens positive/No. of specimens examined	Percentage of positives	No of specimens positive/No. of specimens examined	Percentage of positives
Vermont	VT-1	18/23	78.3	7/27	25.9
	VT-2	7/23	30.4	14/30	46.7
New York	NY-1	21/45	46.7	3/32	9.4
	NY-2	13/42	31.0	20/30	66.7
Pennsylvania	PA-1	17/37	45.9	6/49	12.2
	PA-2	2/43	4.7	NA	NA
	PA-3	NA	NA	3/33	9.1
Maryland	MD-1	21/40	52.5	8/35	22.9
	MD-2	15/30	50	2/26	7.7
Virginia	VA-1	5/25	20	2/21	9.5
	VA-2	14/18	77.8	1/40	2.5
North Carolina	NC-1	16/38	42.1	16/38	42.1
	NC-2	44/61	72.1	1/35	2.9
Florida	FL-1	27/50	54.0	6/43	14
	FL-2	15/28	53.6	3/29	10.3
Total		253/503	50.3	92/468	19.66

NA: not available.

Table 2
Number of specimens (*n*) examined by PCR and sequencing of the 18S rRNA, species or genotype of *Cryptosporidium* that found in pre-weaned calves in each farm

State	Farm	*n*	Cryptosporidium		*C. parvum*		*Cryptosporidium* (Bovine B)		*Cryptosporidium* (deer-like)	
			Positive	Prevalence	Positive	Prevalence	Positive	Prevalence	Positive	Prevalence
VT	VT-1	14	8	57.1	4	28.6	4	28.6	0	0
	VT-2	17	6	35.3	1	5.89	3	17.7	2	11.8
NY	NY-1	25	13	52.0	13	52.0	0	0	0	0
	NY-2	36	9	25.0	9	25.0	0	0	0	0
PA	PA-1	29	11	37.9	11	37.9	0	0	0	0
	PA-2	23	2	8.7	1	4.4	0	0	1	4.4
	PA-3	NA	NA	NA	NA	NA	NA	NA	NA	NA
MD	MD-1	37	11	29.7	10	27.0	0	0	0	0
	MD-2	26	12	46.2	12	46.2	0	0	0	0
VA	VA-1	22	4	18.2	2	9.1	1	4.6	1	4.6
	VA-2	13	10	76.9	8	61.5	2	15.4	0	0
NC	NC-1	33	8	24.24	8	24.2	0	0	0	0
	NC-2	51	37	72.6	32	62.8	3	5.9	2	3.9
FL	FL-1	41	18	43.9	16	39.0	1	2.4	1	2.4
	FL-2	26	12	46.2	11	42.3	0	0	1	3.9
Total		393	161	41.0	138	35.1	14	3.6	8	2.0

Only one calf was infected with *C. anderson* (in MD-1); NA: not available.

Table 3
Number of specimens (*n*) examined by PCR and sequencing of 18S rRNA and species or genotype of *Cryptosporidium* found in post-weaned calves in each farm

State	Farm	*n*	*Cryptosporidium*		*Cryptosporidium* (Bovine B)		*Cryptosporidium* (deer-like)		*C. andersoni*	
			Positive	Prevalence	Positive	Prevalence	Positive	Prevalence	Positive	Prevalence
VT	VT-1	26	9	34.6	2	7.7	7	26.9	0	0
	VT-2	26	13	50.0	12	46.2	2	7.7	0	0
NY	NY-1	28	8	28.6	6	21.4	2	7.1	0	0
	NY-2	25	20	80.0	8	32.0	12	48.0	0	0
PA	PA-1	49	6	12.2	2	4.1	1	2.0	3	6.1
	PA-2	NA	NA	NA	NA	NA	NA	NA	NA	NA
	PA-3	29	12	41.4	9	31.0	2	6.9	0	0
MD	MD-1	35	10	28.6	7	20	0	0	2	5.7
	MD-2	26	7	26.9	2	7.7	0	0	5	19.2
VA	VA-1	21	1	4.8	0	0	0	0	1	4.8
	VA-2	38	3	7.9	0	0	3	7.9	0	0
NC	NC-1	37	10	27.0	4	10.8	6	16.2	0	0
	NC-2	35	5	14.3	5	14.9	0	0	0	0
FL	FL-1	43	5	11.6	3	7.0	1	2.3	1	2.3
	FL-2	29	8	27.6	5	17.2	0	0	3	10.3
Total		447	117	26.2	65	14.5	36	8.1	15	3.4

Only one calf was infected with *C. parvum* (in PA-3); NA: not available.

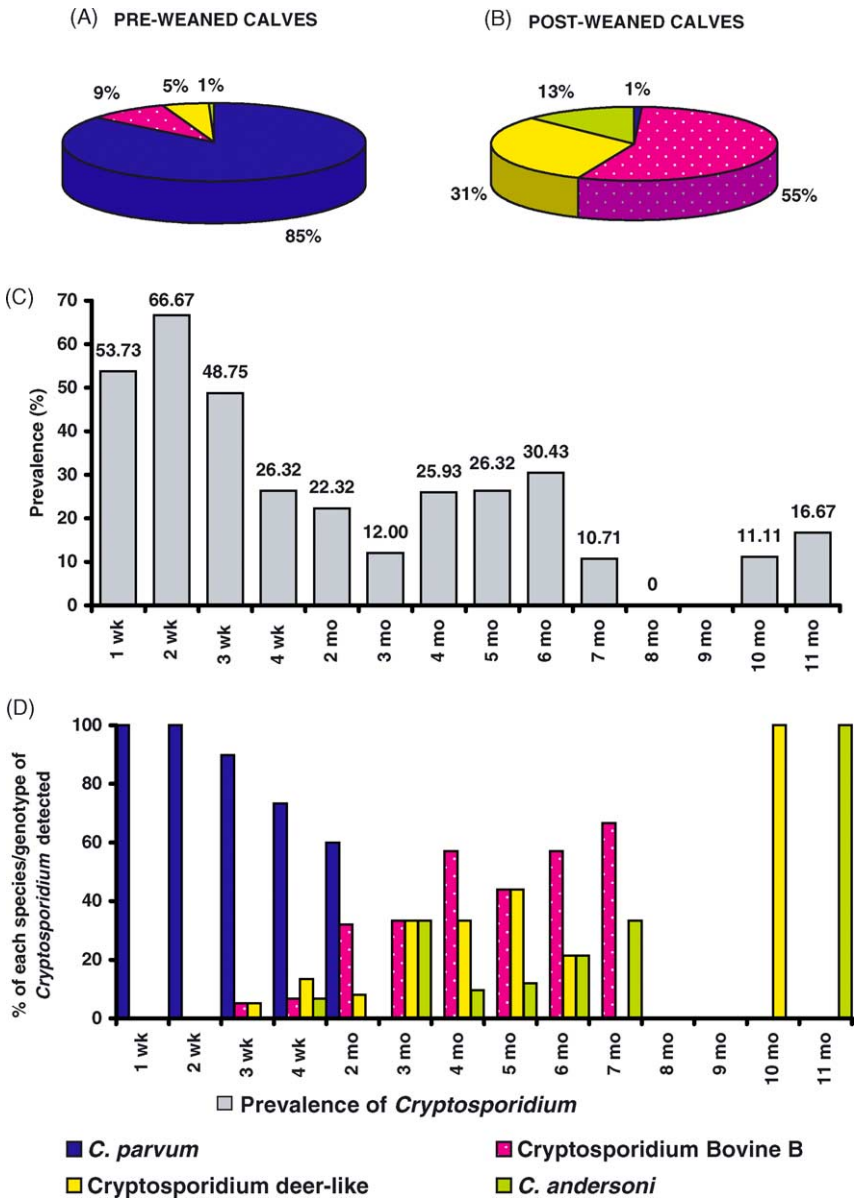

Fig. 1. Colors representing species/genotypes—*C. parvum*: blue, *C. andersoni*: light green, *Cryptosporidium* deer-like: yellow, *Cryptosporidium* Bovine B: light red. (A) Percentage of *C. parvum*, *C. andersoni*, *Cryptosporidium* deer-like, and *Cryptosporidium* Bovine B found in pre-weaned calves. (B) Percentage *C. parvum*, *C. andersoni*, *Cryptosporidium* deer-like, and *Cryptosporidium* Bovine B found in post-weaned calves. (C) Prevalence of *Cryptosporidium*-positive calves detected in calves from 1 week to 11 months of age. (D) Percentage of *C. parvum*, *C. andersoni*, *Cryptosporidium* deer-like, and *Cryptosporidium* Bovine B found in *Cryptosporidium*-infected calves from 1 week to 11 months of age.

Calves were housed under a wide variety of conditions from one farm to another including individual hutches distant from adult cattle, individual wire enclosures within a single large shed, individual solid-wall stalls within a barn, and large pens with 10 or more calves. For example, pre-weaned calves on farms VT-1, VT-2, and NY-2 were housed in individual hutches, on PA-2 were housed as a group in a pen, on NC-2 were housed in individual stalls separated by cement walls covered by a large roof, etc. All post-weaned calves were housed on groups except for those on farm NC-2 that were housed as the pre-weaned calves in individual stalls separated by cement walls.

Feces were collected directly from the rectum of each calf into a plastic specimen cup that was immediately capped, labeled to identify the source based on the ear-tag number, and placed on ice in an insulated container. Feces were transported to the USDA laboratory in Beltsville (Maryland) and processed within 1–3 days of collection.

2.2. Cleaning of specimens from feces

Feces were processed as previously described (Fayer et al., 2000b). Briefly, 15 g of feces from each specimen cup were transferred to a 50 ml centrifuge tube containing approximately 35 ml dH_2O. Each tube was thoroughly mixed, the fecal suspension was passed through a 45 μm screen into a second 50 ml tube and the final volume was adjusted to 50 ml with dH_2O. The tubes were centrifuged at 1800 × *g* for 15 min, the supernatant was discarded, and the pellet was suspended in 25 ml dH_2O and mixed well (Vortex-Genie, Scientific Industries, Bohemia, New York). Twenty-five milliliters of CsCl (1.4 g/l) was added to each tube, the suspension mixed thoroughly, and the tubes subjected to a second centrifugation at 300 × *g* for 20 min. Four milliliters of supernatant, aspirated from the top of each suspension, was transferred to a 15 ml centrifuge tube, and dH_2O added to reach a final volume of 15 ml. Specimens were centrifuged at 1800 × *g* for 15 min and washed twice with dH_2O before the final pellet was suspended in 500 μl of dH_2O. Portions of the 500 μl suspension were examined by immunofluorescence microscopy and molecular analysis as described below.

When the quantity of feces in a specimen was too small to be subjected to the cleaning process, feces were smeared directly onto glass microscope slides and examined for *Cryptosporidium* oocysts by immunofluorescence microscopy. Of 971 specimens 131 were examined this way (110 and 21 specimens in pre- and post-weaned calves, respectively).

2.3. Microscopic examination

A 100 μl aliquot of fecal suspension was transferred to a microcentrifuge tube and washed once with dH_2O. The pellet was resuspended in 50 μl of premixed Merifluor reagent (Meridian Diagnostics, Cincinnati, OH) and 2 μl of suspension was transferred to a well (11 mm diameter) of a 3-well glass microscope slide. The slide was covered with a 24 mm × 50 mm coverslip and the entire well area was examined by fluorescence microscopy at 400× using a Zeiss Axioskop equipped with epifluorescence and an FITC-Texas Red™ dual-wavelength filter.

2.4. DNA extraction

Total DNA was extracted from each 50 μl suspension cleaned of fecal debris using a DNeasyTissue Kit (Qiagen, Valencia, CA). To increase the quantity of recovered DNA, the nucleic acid was eluted in 100 μl of AE buffer (Elution Buffer included in DNeasyTissue Kit).

2.5. 18S rDNA gene amplification and sequencing

A two-step nested PCR protocol was used to amplify the 18S rRNA gene (ca. 830 bp). The fragment of the 18S rRNA gene was amplified by PCR using the following primers previously described by Xiao et al. (1999): 5′-TTCTAGAGCTAATACATGCG-3′ and 5′-CCCATTTCCTTCGAAACAGGA-3′ for primary PCR and 5′-GGAAGGGTTGTATTTATTAGATAAAG-3′ and 5′-AAGGAGTAAGGAACAACCTCCA-3′ for secondary PCR. For the primary PCR step, PCR mixture contained 1× PCR buffer, 3 mM $MgCl_2$, 0.2 mM each dNTP, 2.5 U Taq, 2.5 μl of BSA (0.1 g/10 ml), and 1 μM for each forward and reverse primer in a total of 50 μl reaction volume. A total of 35 cycles, each consisting of 94 °C for 45 s, 59 °C for 45 s, and 72 °C for 1 min, were performed; an initial hot start at 94 °C for 3 min and a final extension step at 72 °C for 7 min were also included. For the secondary PCR step, the PCR mixture was identical except that a concentration of 1.5 mM $MgCl_2$ was used. A total of 40 cycles, each consisting of 94 °C for 30 s, 58 °C for 90 s, and 72 °C for 2 min, were performed; an initial hot start at 94 °C for 3 min and a final extension step at 72 °C for 7 min were also included. PCR products were analyzed on 1% agarose gel and visualized by ethidium bromide staining.

2.6. DNA sequence analysis

PCR products were purified using Exonuclease I/Shrimp Alkaline Phosphatase (ExoSAP-IT™) (USB Corporation, Cleveland, OH). Purified products were sequenced using the same PCR primers in 10 μl reactions, Big Dye™ Chemistries, and an ABI3100 sequencer analyzer (Applied Biosystems, Foster City, CA). Each sample was sequenced in both directions. Sequence chromatograms from each strand were aligned and inspected using Lasergene software (DNASTAR, Inc., Madison, WI).

3. Results

3.1. Prevalence of Cryptosporidium oocysts by microscopy

Microscopic analysis of 971 fecal specimens revealed that 345 (35.5%) were positive for oocysts of *Cryptosporidium*. Based on detection of oocysts, the prevalence of infection for pre- and post-weaned calves on the 15 farms in seven states is shown in Table 1. Positive specimens for *Cryptosporidium* were obtained from all farms studied in 2002 and 2003. Of specimens collected in 2002 from pre-weaned calves, 253 of 503 (50.3%) were positive for

Cryptosporidium. The prevalence of infection for pre-weaned calves ranged from 4.7% on a farm in Pennsylvania (PA-2) to 78.3% on a farm in Vermont (VT-1). Of specimens collected in 2003 from post-weaned calves, 92 (19.7%) of 468 were positive for *Cryptosporidium*. The prevalence of infection for post-weaned calves ranged from 2.5% on a farm in Virginia (VA-2) to 66.7% on a farm in New York (NY-2). Overall, the prevalence of infection based on oocyst detection was greater in pre-weaned than in post-weaned calves. However, there were exceptions at 3 of the 15 farms. At VT-2 and NY-2 the prevalence increased and at NC-1 the prevalence did not change. No clear association was found between housing and prevalence of infection.

3.2. Molecular characterization of Cryptosporidium isolates by PCR and sequencing of a fragment of the 18S rRNA gene

Based on PCR of a fragment of the 18S rRNA gene of *Cryptosporidium*, 278 (33.01%) of the 840 calves examined were positive. For pre-weaned calves the prevalence was 161 (41.0%) of 393 calves examined and for post-weaned calves the prevalence was 117 (26.2%) of 447 calves examined (Tables 2 and 3). Differences in the total number of specimens analyzed by IFA and PCR reflect the use of direct fecal smears for IFA when an insufficient quantity of feces was available for molecular analysis.

For species and genotype identification, 278 specimens were sequenced including all positive PCR products of the 18S rRNA gene. Of these specimens 139 had 100% homology with the *C. parvum* (GenBank accession number: AF093490), 16 had 100% homology with *C. andersoni* (GenBank accession number: AF093496), and in 123 specimens had two additional genotypes of *Cryptosporidium*. In the present study these two genotypes are referred to as *Cryptosporidium* Bovine B and *Cryptosporidium* deer-like genotypes. *Cryptosporidium* Bovine genotype B, identified in 79 specimens, had 100% homology with the Bovine genotype B previously reported from cattle under the GenBank accession number AY120911 (Xiao et al., 2002). *Cryptosporidium* deer-like genotype was identified in 44 specimens and had 99.5% homology with the deer genotype reported from a deer under the GenBank accession number AY120910 (Xiao et al., 2002). The percentage identity between *Cryptosporidium* Bovine B genotype and the deer-like genotype is 98.8%. The nucleotide sequence of the 18S rRNA gene of *Cryptosporidium* deer-like genotype has been deposited in GenBank under the accession number AY587166.

The percentage of each species and genotype of *Cryptosporidium* identified in feces from pre- and post-weaned calves is shown in Fig. 1. For pre-weaned and post-weaned calves, *C. parvum* was identified in 85 and 1% of the specimens, respectively, from the most prevalent species in pre-weaned calves (Fig. 1A) to the least prevalent species in post-weaned calves (Fig. 1B). In contrast, *Cryptosporidium* Bovine B genotype, identified in only 9% of specimens from pre-weaned calves, was the most prevalent (55%) type identified in specimens from post-weaned calves. *C. andersoni*, identified in only 1% of specimens from pre-weaned calves was identified in 13% of specimens from post-weaned calves.

Data relating to the number of calves examined at each farm location, the number of positive specimens at each location, and the species or genotype of *Cryptosporidium* identified by sequencing the 18S rRNA gene are shown for pre-weaned calves in Table 2 and for post-weaned calves in Table 3.

For pre-weaned calves, *C. parvum* was present on all farms examined, with a prevalence ranging from 4.4% for a farm in Pennsylvania (PA-2) to 62.8% for a farm in North Carolina (NC-2) and averaging 35.1% (Table 2). The overall prevalence of *Cryptosporidium* Bovine B and *Cryptosporidium* deer-like genotypes was very low (less than 4%) and *C. andersoni* was identified from only one animal. *Cryptosporidium* Bovine B genotype was identified from six farms in four states, but the only farm with a relatively high prevalence (28.6%) was VT-1. *Cryptosporidium* deer-like genotype was identified from six farms in five states, and *C. andersoni* was identified from only one farm (Table 2).

For post-weaned calves, *Cryptosporidium* Bovine B genotype was present at 12 farms in six states with a prevalence of infection ranging from 0% for both farms in Virginia (VA-1 and VA-2) to 46.2% for a farm in Vermont (VT-2), and averaging (14.5%). *Cryptosporidium* deer-like genotype was present at nine farms in six states with a prevalence of 8%, and *C. andersoni* was present at six farms in four states with a prevalence of 3.4% (Table 3). *C. parvum* was identified from only one calf.

3.3. Prevalence of Cryptosporidium related to the age of calves

The prevalence of *Cryptosporidium* in specimens from calves was determined at weekly age intervals for those under 1 month of age and at monthly age intervals up to 11 months of age (Fig. 1C). Two peaks were observed. The first peak had the highest prevalence (66.7%) and was detected for calves at 2 weeks of age. The second peak prevalence (30.4%) was detected for calves at 6 months of age. In calves older than 6 months the prevalence was much lower.

3.4. Percentage of each species or genotype of Cryptosporidium in calves

The percentage of each species or genotype of *Cryptosporidium* relative to the total number of *Cryptosporidium*-positive calves is shown in Fig. 1D. *C. parvum* constituted 100% of the specimens identified from calves at 1 and 2 weeks of age, and 60–90% of the specimens from calves up to 2 months of age. At 3, 4 and 5–8 weeks (2 months) of age 2, 1 and 8 calves, respectively, were found infected with *Cryptosporidium* Bovine B genotype and 2, 2, and 2 calves, respectively, were found infected with *Cryptosporidium* deer-like genotype. One 4-week-old calf was infected with *C. andersoni*. *C. parvum* was not found in calves 3 months of age or older. In calves 3 months of age or older only *Cryptosporidium* Bovine B and *Cryptosporidium* deer-like genotypes and *C. andersoni* were found. *Cryptosporidium* Bovine B and *Cryptosporidium* deer-like genotypes were first identified from a small number of calves (∼5%) at 3 weeks of age but *Cryptosporidium* Bovine B reached a maximum (∼60%) for calves between 4 and 7 months of age, and the deer-like genotype reached a maximum (∼35%) for calves between 3 and 5 months of age. *C. andersoni*, detected in a single specimen from a calf at 4 weeks of age, reached a maximum (∼30%) for calves between 3 and 7 months of age.

No positive specimens were identified from calves 8 months of age. Specimens were not obtained from calves 9 months of age. Only two positive specimens were identified from calves 10 and 11 months of age, one was *Cryptosporidium* deer-like genotype and the other one was *C. andersoni*.

4. Discussion

Cryptosporidium was not localized to specific farms or states but was present at all 15 farm locations in seven states along the eastern coast of the US from Vermont to Florida. Types of housing appeared to have no influence with regard to prevalence of infection. Approximately 35% of the 971 fecal specimens examined by IFA contained oocysts of *Cryptosporidium* and, similarly, approximately 33% of 840 fecal specimens analyzed by PCR were found positive. The difference in the total number of specimens analyzed by IFA and PCR reflected the use of direct fecal smears for IFA when an insufficient quantity of feces was available for molecular analysis. The number of smeared specimens analyzed by IFA was 110 the first year and 21 the second year. Based on an earlier study detection of oocysts by IFA microscopy in fecal smears is less sensitive than detection after sieving, CsCl density centrifugation, and IFA microscopy (Fayer et al., 2000b). The actual prevalence of infection could be further underestimated with both IFA and PCR because only one fecal specimen was collected per calf. If that specimen was identified as negative during a period when the calf was experiencing intermittent oocyst excretion, the calf would be considered negative. Cumulative prevalence of 100% has been reported for dairy calves at specific locations (Xiao and Herd, 1994; O'Handley et al., 1999; Castro-Hermida et al., 2002b); however, in most cross-sectional studies, the reported infection rate has been lower (Fayer et al., 2000b; Wade et al., 2000; Castro-Hermida et al., 2002a). Two peaks in prevalence were observed in the present study. The first peak in prevalence (66.7%) associated with calves at 2 weeks of age and the second peak in prevalence (30.4%) associated with calves at 6 months of age. Others have also observed this second peak in The Netherlands (Huetink et al., 2001). On a dairy farm with a long history of diarrhea in young calves and *Cryptosporidium* infection, calves 2–7 days of age were excreting oocysts, indicating infection shortly after birth. The percentage of animals excreting oocysts declined after the third week but peaked again at 6 months of age (Huetink et al., 2001). In the present study, in calves older than 6 months prevalence was much lower although *Cryptosporidium*-positive feces were obtained from post-weaned calves from all 15 farms, indicating that infection is common in calves after weaning. The reduction observed in prevalence of *Cryptosporidium* related with the age agreed with most studies that reported the highest prevalence in animals of less than a month of age (Quílez et al., 1996; Sischo et al., 2000; Huetink et al., 2001; Sturdee et al., 2003).

Sequence data for the 18S rRNA gene of *Cryptosporidium* in the 278 positive specimens by PCR identified the species *C. parvum* and *C. andersoni*, as well as the *Cryptosporidium* Bovine B and deer-like genotypes. In the present study, only 1 post-weaned calf (Farm VT-2, 2003) presented a mixed infection with *Cryptosporidium* Bovine B and deer-like genotypes. The lack of detection of mixed infections could be explained since PCR is not able to detect small *Cryptosporidium* subpopulations because of the exponential nature of PCR. Detection of genotypically distinct subpopulations is technically difficult using PCR.

Only *C. parvum* and *C. andersoni* have been reported worldwide as species commonly infecting cattle (Fayer et al., 2000b; Enemark et al., 2002; Peng et al., 2003; Sakai et al., 2003). *C. parvum* has often been found in young calves, whereas *C. andersoni* has always been associated with post-weaned or mature cattle (Fayer et al., 2000b; Huetink et al., 2001; Enemark et al., 2002; Wade et al., 2000; Kvac and Vitovec, 2003). However, the prevalence of *Cryptosporidium* Bovine B and deer-like genotypes in post-weaned cattle

constitutes a new report. Although *Cryptosporidium* Bovine B was reported as an unnamed *Cryptosporidium* sp., it was identified in only two bovine fecal specimens (Xiao et al., 2002). *Cryptosporidium andersoni* and *C. parvum* can be differentiated by size because the oocysts of *C. andersoni* are larger (7.4 μm × 5.6 μm) than those of *C. parvum* (5.0 μm × 4.5 μm) (Lindsay et al., 2000). Another *Cryptosporidium* sp. previously identified in a cow, *C. felis* (Bornay-Llinares et al., 1999), was not found in the present study.

The finding in the present study that *Cryptosporidium* Bovine B and deer-like genotypes were prevalent and widespread appears to stand in contrast to earlier reports. However, most published prevalence studies of *Cryptosporidium* in cattle did not utilize molecular methods that permitted identification of species and genotypes instead they used microscopy methods (e.g. Xiao et al., 1993; Olson et al., 1997; Atwill et al., 1999; O'Handley et al., 1999; Fayer et al., 2000b; Wade et al., 2000; Castro-Hermida et al., 2002a; Kvac and Vitovec, 2003; Ralston et al., 2003). Hence, one cannot be sure which *Cryptosporidium* species was actually present when oocysts were observed in fecal specimens by microscopy. So, without clear diagnostic features that allow the differentiation of *Cryptosporidium* spp. by microscopy, it is not possible to know the precise number of species infecting cattle. In recent years, molecular characterization of *Cryptosporidium* has helped to clarify the confusion in *Cryptosporidium* taxonomy and validate the existence of multiple species (Lindsay et al., 2000; Fayer et al., 2001; Morgan-Ryan et al., 2002; Xiao et al., 2004). The absence of information in the literature about *Cryptosporidium* Bovine B and deer-like genotypes can be explained because: (a) most of the molecular characterization of *Cryptosporidium* isolates for cattle have been part of studies that dealt with improved diagnosis or characterization of isolates, involving neonatal calves most of the time or multiparous cows, and not as part of prevalence studies with bovines of different ages; (b) the size of the oocysts of *Cryptosporidium* Bovine B and deer-like genotypes are similar to those of *C. parvum* not enabling differentiation by microscopy (unpublished data).

Of the four species or genotypes identified in the present study, only *C. parvum* is known to infect humans (Morgan et al., 1999). Therefore, when discussing bovine cryptosporidiosis in a public health context it is necessary to clearly identify these species or genotypes of *Cryptosporidium* or at least the age of animals. Previous reports in which *Cryptosporidium* has been identified only to the genus level or as *C. parvum*, or *C. parvum*-like, based on oocyst morphology without any molecular characterization, must be considered presumptive identification, not confirmatory identification, and therefore could be erroneous or misleading.

The present study documents for the first time a sequential association of species and genotypes of *Cryptosporidium* with cattle as they increase in age and thereby provides a guide for preventing, managing, and tracking sources of cryptosporidiosis. For example, in the present study of 15 farms in seven states, each employing different management conditions, *C. parvum* was associated only with calves less than 2 months of age; *C. parvum* constituted 85% of positive specimens associated with pre-weaned calves, 90% of positive specimens associated with calves 3 weeks of age, and was the only species associated with calves 1–2 weeks of age.

Findings in the present study of two genetically distinct *Cryptosporidium* genotypes found only in cattle, *Cryptosporidium* Bovine B and deer-like genotypes, parallel the findings of two genetically distinct host-specific forms of *Cryptosporidium* isolated from pigs (Enemark

et al., 2003; Ryan et al., 2003) and other host-specific *Cryptosporidium* isolated several other animal species (e.g. Xiao et al., 2002; Jellison et al., 2004). As in the studies with the pig isolates, there is a need to determine the prevalence, health effects, and zoonotic potential of the bovine genotypes utilizing epidemiological studies, experimental transmission to candidate hosts, and multiple gene characterization of isolates.

5. Conclusions

Sequence data for the 18S rRNA gene of *Cryptosporidium* in 278 positives specimens by PCR identified the species *C. parvum*, *C. andersoni* and two unnamed *Cryptosporidium* genotypes Bovine B and deer-like genotype. Results of the present study indicate that the risk of zoonotic infection cannot be determined without molecular characterization of fecal specimens to identify the species implicated in bovine cryptosporidiosis and thereby their zoonotic potential. Past studies in which *C. parvum* was reported in post-weaned calves based on morphology or IFA microscopy of the oocyst stage and not supported by molecular data must be reassessed. The prevalence of the *Cryptosporidium* species/genotypes appeared to be age related. Because calves less than 2 months of age are the predominant population infected with *C. parvum* (zoonotic species), any effort designed to control this infection must be directed primarily at this age group.

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